A 'non-canonical' DNA-binding element mediates the response of the Fas-ligand promoter to c-Myc

Shailaja Kasibhatla*†, Helen M. Beere†, Thomas Brunner‡, Fernando Echeverri and Douglas R. Green†

Cell number is regulated by maintaining a balance between cell proliferation and cell death through apoptosis. Key regulators of this balance include the oncogene product c-Myc, which promotes either entry into the cell cycle or apoptosis [1]. Although the mechanism of c-Myc-induced apoptosis remains unclear, it is susceptible to regulation by survival factors [2,3] and can proceed through the interaction of Fas ligand (FasL) with its receptor, Fas [4]. Activated T lymphocytes are eliminated by an apoptotic process known as activation-induced cell death (AICD), which requires the transcriptional induction of FasL expression [5–7] and sustained levels of c-Myc [8]. The FasL promoter can be driven by c-Myc overexpression, and functional inhibitors of Myc and its binding partner, Max, inhibit the transcriptional activity of the FasL promoter [9,10]. We identified a non-canonical binding site (ATTCTCT) for c-Myc–Max heterodimers in the FasL promoter, which, when mutated, abolished activity in response to c-Myc. Exchange of the canonical c-Myc responsive elements (CACGTG) in the ornithine decarboxylase (ODC) promoter [11] with the non-canonical sequence in the FasL promoter generated an ODC–FasL promoter that was significantly more responsive to c-Myc than the wild-type ODC promoter. Our findings identify a precise physiological role for c-Myc in the induction of apoptosis as a transcriptional regulator of the FasL gene.

Addresses: *Maxim Research, 6650 Nancy Ridge Drive, San Diego, California 92121, USA. †La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, California 92121, USA. ‡Division of Immunopathology, Institute for Pathology, University of Berne, 3010 Berne, Switzerland.

Correspondence: Douglas R. Green; Shailaja Kasibhatla
E-mail: DGreen5240@aol.com; skasibhatla@cytovia.com

Received: **30 May 2000**
Revised: **7 August 2000**
Accepted: **9 August 2000**

Published: **22 September 2000**

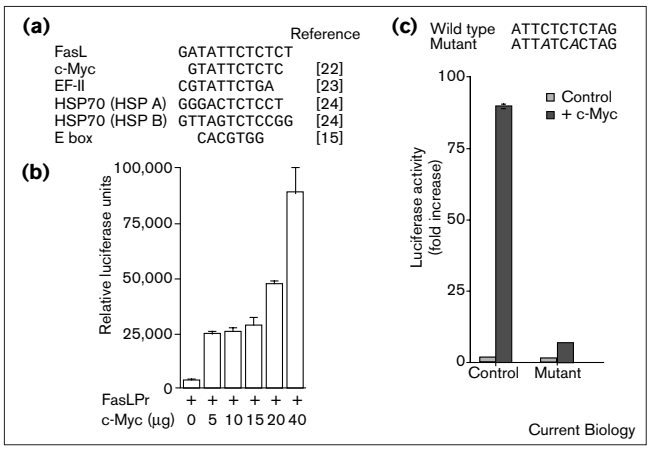
Current Biology 2000, 10:1205–1208

0960-9822/00/$ – see front matter

Results and discussion

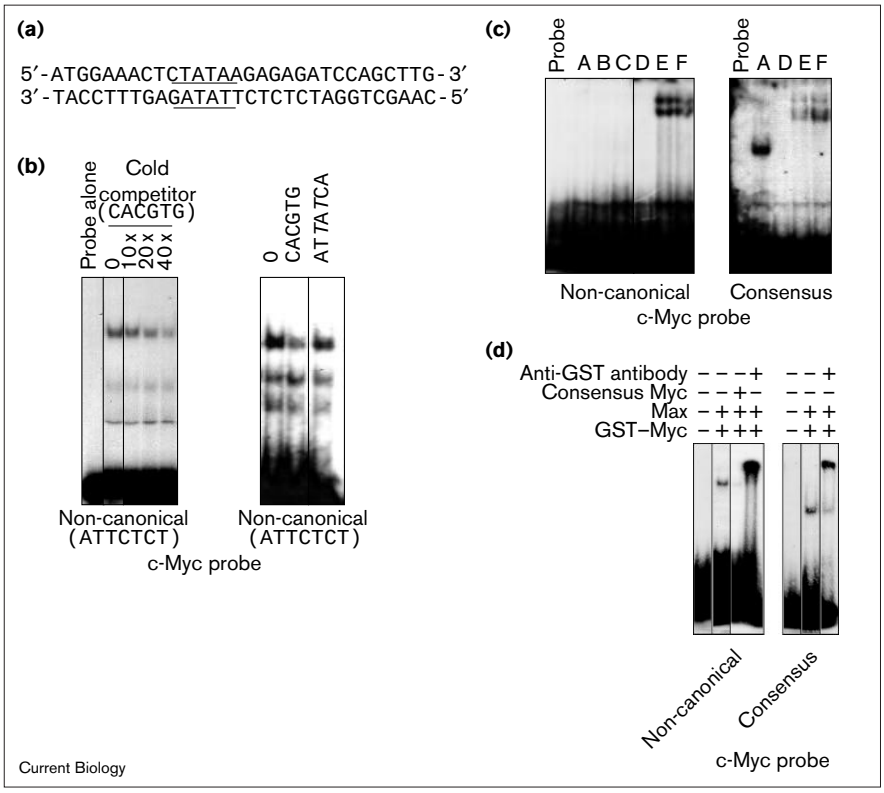
Although there are no canonical Myc-binding sites (E boxes, CACGTG) in the region 1.2 kb upstream of the FasL gene [12], examination of the FasL promoter sequence revealed a site that has been described as a putative binding site for c-Myc in a number of genes, including EF-II, Hsp70 and c-Myc itself (Figure 1a). To examine whether this element was responsive to c-Myc and could drive expression of the FasL promoter, we coexpressed the 1.2 kb FasL promoter reporter construct [12] with a c-Myc expression vector in Jurkat T cells. We observed a significant activation of the FasL promoter in response to elevated expression of c-Myc (Figure 1b). To verify the specificity of this observation, we generated a mutant FasL promoter construct containing base changes within the putative Myc-binding region (ATT*A*TC*A*C-TAG, the substituted bases are italicized). This mutation effectively eliminated the response of the FasL promoter to c-Myc co-expression (Figure 1c) and to T-cell activation signals mimicked by phorbol 12-myristate 13-acetate (PMA) plus ionomycin (data not shown).

Figure 1



Identification of a c-Myc-responsive element in the FasL promoter. **(a)** Comparison of the consensus Myc-binding site (E box) with a number of previously identified non-canonical Myc-responsive elements [15,22–24], including the putative site in the FasL promoter. **(b)** Overexpression of c-Myc enhances FasL promoter activity. Jurkat cells were transiently co-transfected with the 1.2 kb FasL promoter reporter construct (FasLPr) and increasing concentrations of c-Myc expression vector or empty vector (pSP271) as indicated. Data are expressed as mean relative luciferase units (± SD) and normalized to β-galactosidase activity. **(c)** A mutation in the c-Myc-binding site decreases c-Myc responsiveness of the FasL promoter. Jurkat cells were transiently co-transfected with a mutant reporter construct and increasing amounts of c-Myc expression vector as indicated. Data are expressed as fold increase in luciferase activity. The results in (b,c) are representative of at least three independent experiments, each carried out in triplicate. The error bars indicate SD.

Figure 2



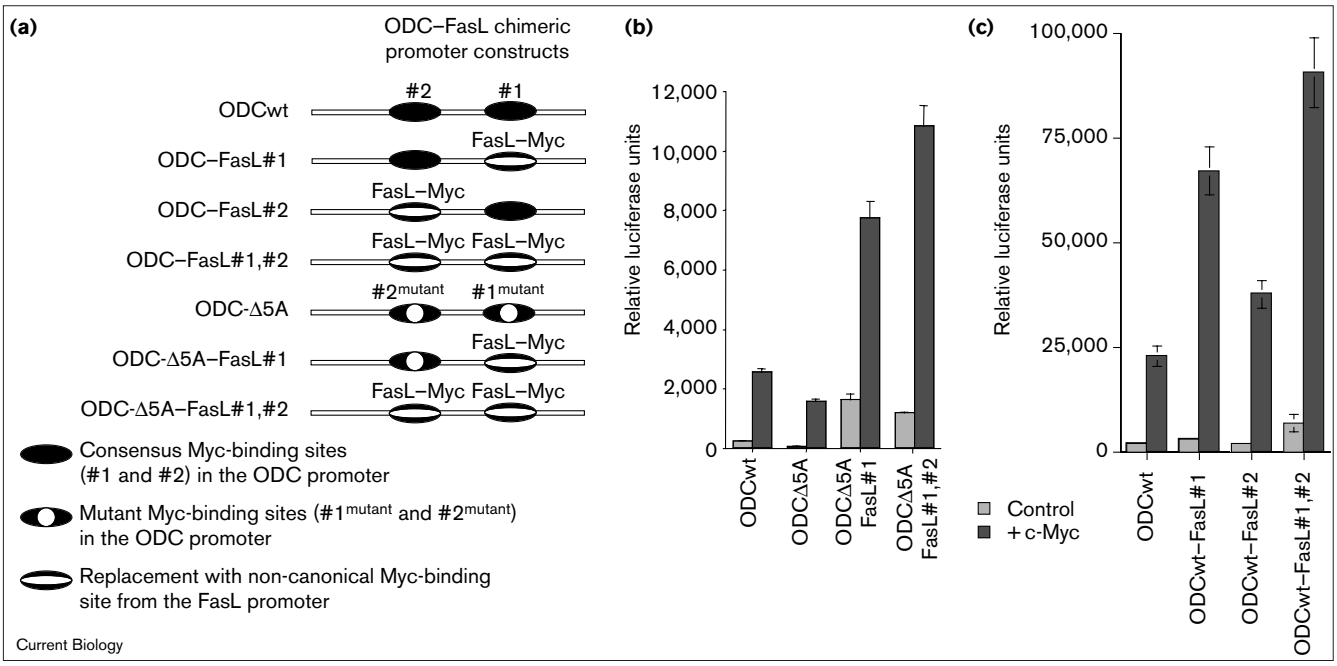
Recombinant Myc–Max heterodimers, but not c-Myc alone, bind directly to the non-canonical Myc-binding site in the FasL promoter. **(a)** Sequence of the FasL promoter region containing the putative c-Myc responsive element (underlined) at −120 bp upstream of the translation start site. This sequence was used as a double-stranded oligonucleotide for the EMSA experiments. **(b)** Binding of nuclear proteins to the non-canonical c-Myc-binding site in the FasL promoter. Left, nuclear extracts were incubated with the radiolabeled oligonucleotide described in (a) and binding was competed with increasing amounts of the unlabelled consensus oligonucleotide (CACGTG). Right, nuclear extracts were incubated with radiolabeled probe (ATTCTCT) described in (a) and binding was competed with oligonucleotide containing either a consensus Myc site or a mutated Myc site in the FasL promoter. **(c)** Binding of recombinant GST–Myc and GST–Max to the Myc-binding site in the FasL promoter. Left, the ^{32}P-labeled oligonucleotide from (a) was incubated with GST–Myc and GST–Max proteins as indicated and binding was assessed by EMSA. Right, comparison of binding of recombinant GST–Myc and GST–Max to the consensus Myc-binding site. Incubations are as follows: A, GST–Myc (100 ng); B, GST–Myc (200 ng); C, GST–Myc (500 ng); D, GST–Max (100 ng); E, GST–Myc (200 ng) + GST–Max (100 ng); F, GST–Myc (200 ng) + GST–Max (200 ng); Probe (probe alone). **(d)** Binding of recombinant GST–Myc and Max (no GST tag) to the canonical and non-canonical c-Myc-binding sites can be supershifted using an anti-GST antibody.

To determine whether the site we have identified in the FasL promoter binds c-Myc, we generated a double-stranded oligonucleotide probe containing this region of the FasL promoter (Figure 2a). Proteins in nuclear extracts isolated from the T-cell hybridoma line A1.1 bound this probe in an electromobility shift assay (EMSA; Figure 2b). This binding was effectively competed with an excess of unlabelled oligonucleotide containing a canonical c-Myc-binding sequence, CACGTG (Figure 2b, left). However, an oligonucleotide containing a mutated FasL promoter sequence (ATT*A*TC*A*) was unable to compete for binding (Figure 2b, right). This observation correlated with functional data showing that this mutation, when introduced into the 1.2 kb FasL promoter, abolished the responsiveness of the FasL promoter construct to c-Myc (Figure 1c).

Previous observations had indicated a potential role for Max in the activity of c-Myc in regulating FasL transcription and induction of AICD [9,10]. To examine more directly the possibility that the site we have identified in the FasL promoter binds to c-Myc and that its binding partner, Max, may also be required, we used glutathione-S-transferase (GST) fusions of Myc and Max proteins in the EMSA. Myc and Max proteins bound as heterodimers to both the non-canonical and the consensus site in the FasL promoter (Figure 2c). However, c-Myc alone was only able to recognise and bind the consensus sequence. Max alone was unable to bind either the non-canonical or the consensus Myc sites. These data indicate that the non-canonical c-Myc-binding motif selectively binds Myc–Max heterodimers. Confirmation that recombinant GST–Myc and Max (no GST tag) heterodimers formed a complex with oligonucleotides containing canonical or non-canonical Myc sites was shown by the detection of a supershifted complex using an anti-GST antibody (Figure 2d).

We reasoned that if the sequence in the FasL promoter is a *bona fide* Myc-responsive element, then it might substitute for Myc-responsive elements in other promoters. The ODC promoter responds to c-Myc and contains two canonical Myc-binding sites [11]. We generated constructs in which the Myc-binding sites in the ODC promoter (denoted #1 and #2) were mutated and/or replaced with the non-canonical c-Myc sequence from the FasL promoter (Figure 3a). As shown in Figure 3b, the wild-type ODC promoter (ODCwt) was induced by co-expression

Figure 3



Replacement of the consensus Myc-binding site(s) in the ODC promoter with the non-canonical Myc-binding sites from the FasL promoter sustains Myc responsiveness. **(a)** Constructs. One or both of the consensus Myc-binding sites (#1 and #2) in the wild-type ODC promoter (ODCwt) and the mutant Myc-binding sites in the ODCΔ5A promoter [11] were replaced with the non-canonical Myc-binding site from the FasL promoter to generate a series of promoter reporter constructs. **(b)** The non-canonical Myc-binding site from the FasL promoter sustains Myc responsiveness in the ODC promoter. Replacement of one (ODCΔ5A–FasL#1) or both (ODCΔ5A–FasL#1,#2) the mutant c-Myc elements in the ODCΔ5A promoter with the non-canonical Myc sequence from the FasL promoter. Jurkat T cells were transiently co-transfected with these constructs in the presence or absence of a c-Myc expression vector and luciferase activity assessed. **(c)** Replacement of one (ODCwt–FasL#1 or ODCwt–FasL#2) or both (ODCwt–FasL#1, #2) the consensus c-Myc elements in the ODCwt promoter with the non-canonical Myc sequence from the FasL promoter. Jurkat cells were transiently co-transfected with the ODCwt promoter chimeras in the presence or absence of a c-Myc expression construct. Data in (b,c) are expressed as relative luciferase units and normalised to β-galactosidase activity. These results are representative of at least three independent experiments, each carried out in triplicate. The error bars indicate SD.

with c-Myc, and mutation of both of the Myc-binding sites (ODCΔ5A) decreased this responsiveness [11]. When one (ODCΔ5A–FasL#1) or both (ODCΔ5A–FasL#1,#2) the mutated sites were replaced with the non-canonical Myc sequence, the Myc responsiveness of the ODC promoter was restored to a level greater than that seen with the wild-type promoter. We conclude that the non-canonical Myc-binding site from the FasL promoter can functionally replace canonical Myc-binding sites in an established Myc-responsive promoter.

We also performed the experiment by sequential exchange of the two wild-type sequences in the ODC promoter with the non-canonical binding sequence from the FasL-promoter (ODCwt–FasL#1, ODCwt–FasL#2 and ODCwt–FasL#1,#2). This generated a series of reporter constructs that all exhibited an enhanced responsiveness to c-Myc (Figure 3c) compared with ODCwt. These data further implicate this region of the FasL promoter as a Myc-responsive element.

We have previously shown that c-Myc is required for AICD and FasL expression in T cells and T-cell hybridomas [8–10]. Together with the data presented here, our results implicate a non-canonical site in the FasL promoter in binding the Myc–Max heterodimer, and which is necessary but not sufficient for expression of the FasL gene. Other transcription factors that are activated upon T-cell receptor (TCR) ligation and that participate in FasL gene expression include NF-AT, EGR-2 and NF-kB [13]. This activation-induced FasL mRNA expression proceeds in the presence of cycloheximide [14], which suggests that the essential factors (including c-Myc) act directly to drive the FasL promoter rather than through activation and induced expression of other transcription factors. Together, these transcription factors co-operate to optimally induce activation of the FasL promoter, leading to gene expression. When FasL is expressed, it ligates its cell-surface receptor Fas, which in turn engages the appropriate intracellular machinery, culminating in activation of caspases and apoptotic death of the cell [5–7].

The site we have identified as a Myc-responsive site in the FasL promoter is similar or identical to other sites that have previously been suggested to be Myc responsive (see Figure 1a). This site, however, is only remotely similar to the well-established E box present in a number of Myc-responsive promoters (including the ODC promoter) [11] that were identified by direct binding of Myc to randomized oligonucleotides [15]. Why was the site that we have identified not detected as Myc binding in such studies? One possible answer is suggested by the observation shown in Figure 2c in which oligonucleotides containing this sequence failed to bind recombinant c-Myc (or did so very poorly), but instead bound recombinant Myc–Max heterodimer effectively. Notably, one of our earlier studies described the requirement for Myc–Max heterodimers in AICD [10]. This difference in binding by Myc versus Myc–Max (Figure 2c) may help account for the difference in detection of sites such as we have identified versus the more conventional E-box-related sites.

We recently reported that transforming growth factor-β (TGF-β) suppresses the transcriptional activation of FasL and AICD by the direct inhibition of c-Myc expression [16]. Furthermore, interleukin-2 (IL-2) sensitizes T cells to AICD and promotes elevated c-Myc expression [17], making it tempting to speculate that c-Myc may contribute to this sensitization process. These observations support our belief that c-Myc plays a key role in regulating FasL expression under physiologically relevant situations.

Why should FasL expression in lymphocytes be linked to the expression and function of c-Myc? Only activated, proliferating cells, and not resting lymphocytes, express c-Myc. As clonal expansion forms the basis of immune responses, it is only the proliferating cells that may represent a threat to the body should they happen to be autoreactive (or even hyper-reactive, as these will also cause extensive bystander damage). Re-stimulation of activated cells therefore induces FasL, which in turn serves to check cellular expansion by induction of apoptosis. Alternatively (and non-exclusively), activated, proliferating lymphocytes that take on effector functions do so, in part, through the expression of FasL (except in this case they are resistant to Fas-mediated death), which functions to kill Fas-expressing cells with which they come in contact. By limiting this expression to proliferating cells, it serves as a fail-safe mechanism to ensure that once the cells cease to express c-Myc (for example, at the cessation of the response) expression of this lethal molecule will also cease.

Supplementary material

Supplementary material including full Materials and methods is available at http://current-biology.com/supmat/supmatin.htm.

References

1. Harrington EA, Fanidi A, Evan GI: **Oncogenes and cell death.** *Curr Opin Genet Dev* 1994, **4:**120-129.
2. Harrington EA, Bennett MR, Fanidi A, Evan GI: **c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines.** *EMBO J* 1994, **13:**3286-3295.
3. Bissonnette RP, Echeverri F, Mahboubi A, Green DR: **Apoptotic cell death induced by c-myc is inhibited by bcl-2.** *Nature* 1992, **359:**552-554.
4. Hueber AO, Zornig M, Lyon D, Suda T, Nagata S, Evan GI: **Requirement for the CD95 receptor-ligand pathway in c-myc-induced apoptosis.** *Science* 1997, **278:**1305-1309.
5. Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, *et al.*: **Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation induced apoptosis in T-cell hybridomas.** *Nature* 1995, **373:**441-444.
6. Dhein J, Walczak H, Baeumler C, Debatin K-M, Krammer PH: **Autocrine T-cell suicide mediated by APO-1/(Fas/CD95).** *Nature* 1995, **373:**438-441.
7. Ju S-T, Panka DJ, Cui H, Ettinger R, El-Khatib M, Sherr DH, *et al.*: **Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation.** *Nature* 1995, **373:**444-448.
8. Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP, Green DR: **Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas.** *Science* 1992, **257:**212-214.
9. Brunner T, Kasibhatla S, Pinkoski MJ, Frutschi C, Yoo NJ, Echeverri F, *et al.*: **Expression of Fas ligand in activated T cells is regulated by c-Myc.** *J Biol Chem* 2000, **275:**9767-9772.
10. Bissonnette RP, MacGahon AJ, Green DR: **Functional Myc-Max heterodimer is required for activation-induced apoptosis in T cell hybridomas.** *J Exp Med* 1994, **180:**2413-2418.
11. Bello-Fernandez C, Packham G, Cleveland JL: **The ornithine decarboxylase gene is a transcriptional target of c-Myc.** *Proc Natl Acad Sci USA* 1993, **90:**7804-7808.
12. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR: **DNA damaging agents induce expression of fas-ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kB and AP-1**. *Mol Cell* 1998, **1:**543-551.
13. Pinkoski MJ, Green DR: **Fas ligand, death gene.** *Cell Death Differ* 1999, **6:**1174-1181.
14. Brunner T, Yoo NJ, Griffith TS, Ferguson TA, Green DR: **Regulation of CD95 ligand expression: a key element in immune regulation.** *Behring Res Comm* 1997, **97:**161-174.
15. Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H: **Sequence-specific DNA binding by the c-Myc protein.** *Science* 1990, **250:**1149-1151.
16. Genestier L, Kasibhatla S, Brunner T, Green DR: **Transforming growth factor beta1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via downregulation of c-Myc.** *J Exp Med* 1999, **189:**231-239.
17. Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J, Zheng L: **Mature T lymphocyte apoptosis – immune regulation in a dynamic and unpredictable antigenic environment.** *Annu Rev Immunol* 1999, **17:**221-253.
18. Blackwood EM, Eisenman RN: **Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc.** *Science* 1991, **251:**1211-1217.
19. Grandori C, Mac J, Siebelt F, Ayer DE, Eisenman RN: **Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites *in vivo*.** *EMBO J* 1996, **15:**4344-4357.
20. Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S: **Human Fas ligand: gene structure, chromosomal location and species specificity.** *Int Immunol* 1994, **6:**1567-1574.
21. Tillman JB, Crone DE, Kim HS, Sprung CN, Spindler SR: **Promoter independent down-regulation of the firefly luciferase gene by the T3 and T3 receptor in CV1 cells.** *Mol Cell Endocrinol* 1993, **95:**101-109.
22. Ariga H, Imamura Y, Iguchi-Ariga SM: **DNA replication origin and transcriptional enhancer in c-myc gene share the c-myc protein binding sequences.** *EMBO J* 1989, **8:**4273-4279.
23. Hann SR, Dixit M, Sears RC, Sealy L: **The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site.** *Genes Dev* 1994, **8:**2441-2452.
24. Taira T, Iguchi-Ariga SM, Ariga H: **A novel DNA replication origin identified in the human heat shock protein 70 gene promoter.** *Mol Cell Biol* 1994, **14:**6386-6397.